ACS SYMPOSIUM SERIES 621

Immunoassays for Residue Analysis

Food Safety

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Developed from a symposium sponsored
by the Division of Agriculture and Food Chemistry
at the 209th National Meeting
of the American Chemical Society,
Anaheim, California,
April 2–7, 1995

American Chemical Society, Washington, DC 1996

Chapter 34

Coupling Enzyme Immunoassay with Supercritical Fluid Extraction

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The coupling of enzyme immunoassay with supercritical fluid extraction (SFE) is an attractive technique for analysts faced with decreasing the use of hazardous solvents, due to their adverse impact on the environment. This chapter will describe the development of supercritical fluid extraction techniques which can be combined with enzyme immunoassays for the detection of pesticide residues and similar toxicants in food and environmental samples. The use of static versus dynamic SFE will be contrasted with respect to speed of analysis, equipment requirements, and quantitative vs. qualitative analysis. Detection of the presence of pesticides in meat matrices was accomplished using different commercial test kits. Removal of various interferences from the sample extract prior to EIA is necessary to achieve quantitative results, due to the presence of lipid coextractives in the extract. The above techniques have been successfully employed to determine pesticide residue content in meat products and other matrices below their specified tolerance limit set by regulatory agencies.

The use of enzyme immunoassay (EIA) for the determination of trace levels of toxicants in food and environmental matrices is now well documented in the literature (*1,2*). EIA offers the possibility of rapid determination, both qualitatively and quantitatively, of a variety of pesticides, drugs, and mycotoxins that can contaminate the food supply of the United States (*3–6*). Many EIA protocols also utilize aqueous solutions or a minimal amount of organic solvent while performing the test assay; making them environmentally-compatible in both a laboratory or field/plant setting.

Supercritical fluid extraction (SFE), a sample preparation technique, has developed concurrently in time with EIA over the past ten years (*7*). Performing SFE with supercritical carbon dioxide (SC–CO_2), can eliminate many of the problems associated with the use of organic solvents in extractions, namely flammability,

toxicity, cost of purchase and disposal, and adverse impact on the environment. It also has been demonstrated that analytical SFE can yield shorter extraction times and more precise recovery of analytes relative to Soxhlet extractions for analyte concentrations at the parts per billion (ppb) level; i.e., pesticides (*8,9*), mycotoxins (*10*), and environmental contaminants (*11,12*).

Coupling SFE with analysis techniques, such as EIA or capillary electrophoresis (CZE), which use minimal amounts of solvents, provides the analyst with tandem methodologies that have several mutual advantages. Since both techniques (SFE and EIA) are environmentally-compatible, and if the two methods could be coupled, then on-site testing becomes possible, eliminating the need to transport samples to a laboratory with the associated loss of time. Analysis time can further be minimized if both techniques can be performed relatively fast. Within limits, EIA offers the possibility of screening large numbers of samples qualitatively for the presence of target analytes. This eliminates the need for an analyses that uses expensive equipment and/or reagents, and the associated labor costs to perform sophisticated instrumental analysis. For these reasons, we have conducted research on behalf of the USDA's Food Safety and Inspection Service (FSIS) for the past two years to couple SFE with EIA for the analysis of pesticide residues in meat products (*13,14*).

Several research teams have complemented our research efforts and utilized SFE for specific purposes. Earlier research by Wong *et al.* (*15*) indicated that SFE could be combined with EIA for the determination of parathion and its oxidation product, 4-nitrophenol, in environmental matrices such as soil. Further research by Lopez-Avila and Van Emon (*16,17,* See chapter by Lopez-Avila *et al.*, this volume) has illustrated the general applicability of the SFE/EIA technique to the determination of pesticides and polychlorinated biphenyls in soils, and recently these investigators have reported an extension of their methods for the analysis of drugs in tissue samples (*18*). The recent availability of several portable analytical SFE modules (*19–21*) also should further the application base for SFE/EIA in field or plant environments.

In this chapter, we shall emphasize factors that are crucial for the successful coupling of SFE with EIA since our expertise lies first and foremost in analytical SFE. The reported methods development research has been centered principally on applying SFE/EIA for the analysis of pesticides in foods, such as meats, containing a large amount of potential coextractives (fats) that also dissolve in SC–CO_2. Hence, we will describe here the development procedure for SFE/EIA assays, emphasizing ways of minimizing coextractives, or dealing with their effect in the subsequent EIA test. However, some basic principles of SFE need to be understood by the reader and these are discussed below.

Some Basic Principles of SFE

Analytical SFE exhibits many advantages over conventional sample preparation methods for the isolation of toxicant residues from a variety of sample matrices. It has been demonstrated that quantitative extractions of many environmental toxicants can be achieved via SFE for environmental pollutants, pesticides, and naturally occurring toxicants, such as mycotoxins. SFE permits extractions to be accomplished in a shorter time interval, and with better precision, relative to Soxhlet extraction for

trace analytes down to the part-per-billion level. Analytical SFE is not normally concerned with the extraction of large quantities of material; consequently, it can be applied for the analysis of a wide variety of analytes (encompassing a large polarity range) at trace levels in different sample matrices.

Figure 1 depicts the SFE process, with an accompanying graph depicting the solubility of a frequently studied solute, naphthalene, in SC–CO_2 as a function of pressure and temperature. The SFE process is inherently simple: the components or matrix to be extracted are placed in the extraction vessel, a compressor or pump supplies the supercritical fluid to the extraction vessel, the dissolved solutes pass through a pressure reduction device (valve or restrictor) and are collected in some type of separator device (solvent, sorbent, empty vessel), by a reduction in pressure and/or temperature, usually to ambient conditions. The choice of extraction and/or separation conditions is to a first approximation, based on the solubility of the target analyte(s) in the supercritical fluid. An example of this principle for naphthalene in SC–CO_2 is shown in the right hand portion of Figure 1. Here naphthalene will dissolve to the extent of 5.2 mole% in SC–CO_2 at 300 bar and approximately 55 °C, conditions which typically would be used for the extraction step (E_1). A partial reduction in naphthalene's solubility in SC–CO_2 can then be affected in the separator (S_1, S_2) by reducing the pressure to 90 atm at approximately 45 °C, which yields a naphthalene concentration of 0.2 mole%, or alternatively to 1.2 mole% by reducing the temperature along the isobar to approximately 20 °C. Obviously, even greater reductions in solubilities can be achieved by performing the separation at ambient conditions. This should result in a good recovery of naphthalene, providing the trapping (separator) device is optimized for collecting the analyte.

Perhaps the key parameter in understanding the efficacy of SFE is the effect of extraction pressure on solute solubility in the supercritical fluid phase. Figure 2 illustrates a typical solubility curve for the solute, naphthalene, in SC–CO_2 under isothermal conditions. Here the onset of naphthalene's solubility in SC–CO_2; i.e., its "threshold pressure" (*22*), occurs slightly before 75 atm. As can be seen in Figure 2, the solubility of naphthalene rises precipitously over a narrow interval of pressure, resulting in a relatively high solubility for this particular solute at pressures under 200 atm. This behavior is fairly typical for many solutes undergoing SFE, but can be moderated substantially by the type of matrix from which the solute is extracted (*23*).

Similar solubility trends for lipid solutes (triglycerides) in SC–CO_2 have been recorded (*24*). Consequently, if there is not a sufficient difference in the threshold pressures of a target analyte (i.e., naphthalene) and a typical coextractive, such as triglycerides (i.e., fats/oils); then total separation is impossible, resulting perhaps in undesirable interferences being present in the final analytical assay method (EIA). Differences in solute threshold pressures and solubilities in supercritical fluids are attenuated by significant differences in the chemical structure (i.e., polarity) of solutes, their relative volatilities, or large differences in their respective molecular masses. Unfortunately in many SFEs, the above conditions with respect to the solutes being extracted do not exist, resulting in some contamination of the desired analyte with interfering coextractives. Hence, the resultant extract may or may not require some degree of cleanup, depending on the specificity of the analysis method for the analyte(s). This step also may be ignored if the coextractives do not interfere in the

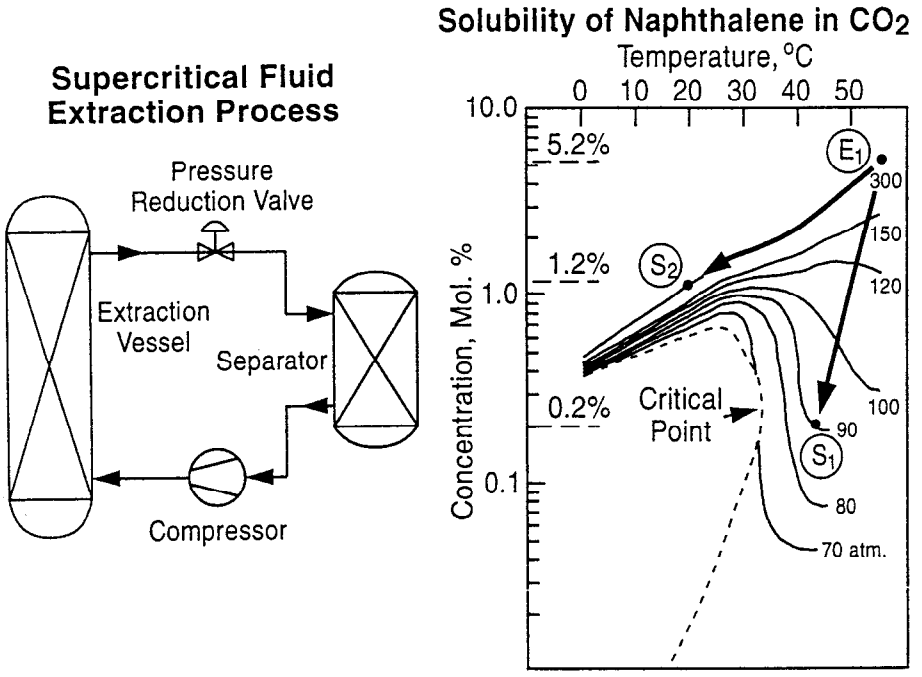

Figure 1. The basic SFE process and solubility of naphthalene in SC–CO_2 as a function of temperature and pressure (E_1 = extraction conditions; S_1, S_2 = separator conditions).

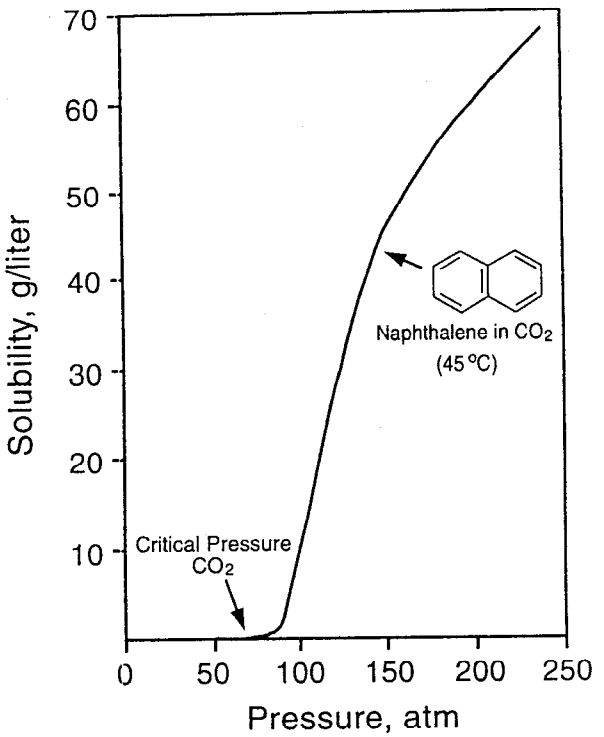

Figure 2. Solubility of naphthalene in SC–CO_2 as a function of pressure at 45 °C.

final assay method or contribute to contamination of the measuring device (instruments).

Figure 3 shows the solubility curve for the pesticide, alachlor, in SC–CO_2 as a function of pressure at 55 °C. The threshold pressure in this case is well below 100 atm, and alachlor's solubility in CO_2 is substantial; approximately 20 g/100 g of CO_2 at 250 atm. This is more than enough analyte solubility for detection by most EIA techniques, suggesting that high extraction pressures would not be required for most SFE/EIA couplings based on solubility considerations alone. Such an observation opens up the possibility of applying SFE/EIA for the qualitative assay of many analytes at trace levels, since high analyte solubilities in the extractant phase are not required for trace analysis. In addition, the extraction of trace analytes does not require a large quantity of CO_2 at extremely high pressures to affect removal of the analyte from some sample matrices, opening the way for the use of a small, portable extraction device that does not require access to a cylinder source for fluid supply or a large, cumbersome electrically- or pneumatically-driven pump to deliver a compressed fluid to the extractor proper.

Approaches for Coupling SFE with EIA

As noted in the introduction, one of the major advantages in using immunoassay is the ability to apply the technique in the field for rapid quantification of contaminants, such as pesticides or mycotoxins. This seminal goal has guided our approach to coupling SFE with EIA, and we shall describe in this section the generic experimental approach which has been utilized in developing several novel devices applicable to field assay work. Further detail of the associated experimental apparatus and technique are described in the literature (*13, 14*) and the emphasis here will be on the integration of the two techniques, including several new methods not previously described.

The design of the experimental apparatus and protocols embodied several features associated with both techniques. Water was chosen as the collection solvent because most of current EIA kits are designed to function in aqueous media. In addition, water also is a compatible solvent for use in a food processing plant environment. Initial studies also utilized low extraction pressures to limit the extraction of lipids which might interfere in the EIA. Dry ice was examined as a CO_2 source since it is readily available and could be used in a processing plant. Although the purity of most dry ice would be inadequate for some SFE processes when coupled off- or on-line with classical detection methods (i.e., ECD/GC analysis); however, this proved to be a negligible problem with EIA detection methods due to their high specificity. We also have found that dry ice can aid in homogenizing tissue samples as shown by Benville and Tindle (*25*).

The initial "static" system used in our SFE/EIA studies is shown in Figure 4. This system used an extraction cell that was fabricated out of high pressure tubing (Autoclave Engineers, Erie, PA) (*13*), sealed on one end to provide a reservoir, not only for the sample, but also for the extraction fluid (CO_2). Dry ice was then added to cool the cell, followed by additional dry ice for use as the extraction fluid (CO_2). At this point the sample was introduced into the cell before capping the vessel with an assembly consisting of a pressure gauge, on/off valve, and restrictor. Pressure for the

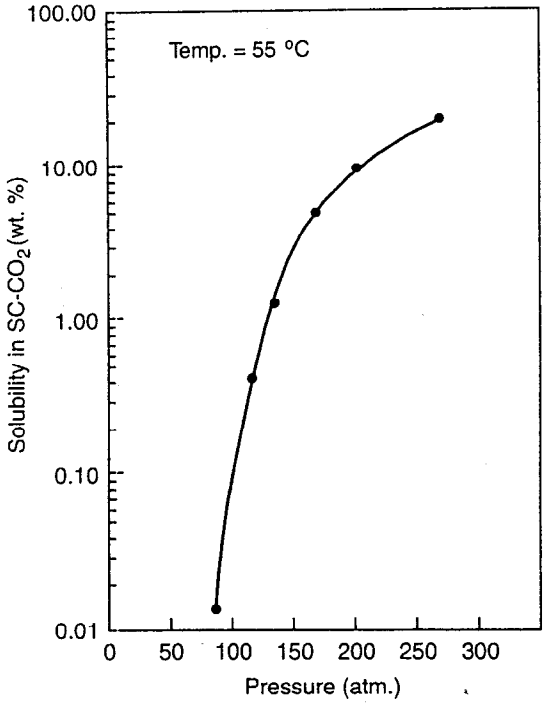

Figure 3. Solubility of alachlor in SC–CO_2 as a function of pressure at 55 °C.

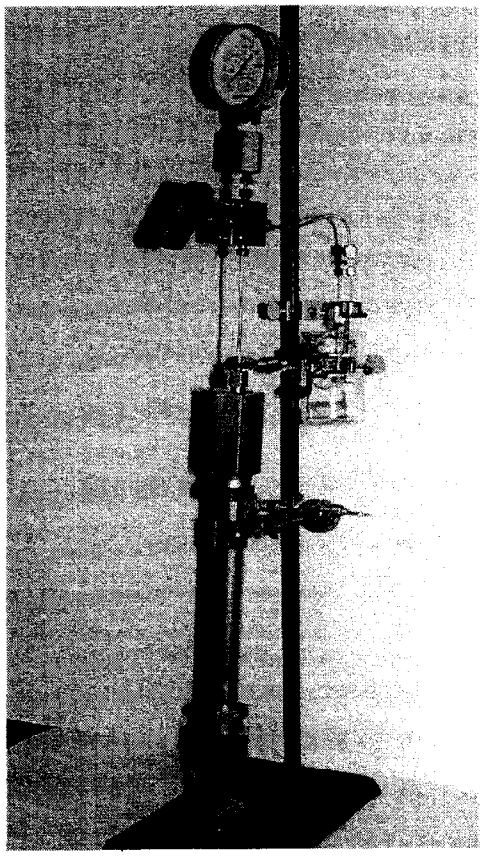

Figure 4. Static, pumpless SFE system.

extraction was developed by means of applying heat to the extraction cell, thereby expanding the CO_2 in a fixed volume. Static holds of 15 min at the developed pressures (86–118 atm) have been sufficient to extract enough analyte for qualitative determination of pesticide residues using commercial EIA kits. The vessel is simply vented at the end of this single stage SFE through an on/off valve into a collection vial filled with water via a 0.127 mm id restrictor made of PEEK tubing. Freezing of the water due to the Joule–Thomson expansion effect of CO_2 was moderated by placing the extract collection vial containing 5 mL of water into another beaker containing water. Both the beaker and the extraction cell can be heated with the aid of heating mantles (Glas-Col Company, Terre Haute, IN).

Using the above described system greatly simplifies the number of components needed in the field for conducting the qualitative SFE. Results for the determination of alachlor down to a 5 ppb level in fortified lard and poultry tissue are given in Table I, using a Resi-I Quant alachlor kit (Immunosystems, Scarborough, ME) for detection of the analyte. Similar experiments also were conducted using CO_2 extraction with a small quantity of methanol added as a cosolvent to the extraction cell.

Table I. Static SFE/EIA Screening Results.

Sample	Alachlor Detected[a]
Dry Ice (x 3)	–
Lard (x 3) (0.5–2.1 g)	–
Alachlor, (20 ng)	+
Lard (g), spiked with alachlor (ppb)	
1.9 g, 5 ppb Alachlor	–
2.4 g, 10 ppb Alachlor	+
2.5 g, 25 ppb Alachlor	+
1.1 g, 50 ppb Alachlor	+
2.3 g, 50 ppb Alachlor	+
0.5 g, 100 ppb Alachlor	+
Poultry Tissue	
10.1 g, No spike	–
12.0 g, 4.2 ppb Alachlor	+

[a]The + = pesticide detected (concentration of alachlor in collection water is above detection limit); the – = pesticide not detected (concentration of alachlor in water is below detection limit).

As shown in Table II, alachlor spikes could be detected in both lard and liver matrices, although sensitivity of the EIA kit was inhibited somewhat by the presence of methanol.

TABLE II. SFE/EIA Screening Results on Lard and Bovine Liver Samples (with Methanol Addition).

Sample	Alachlor Detected
No Sample	–
0.68 g Lard	–
0.52 g Lard, 100 ppb Alachlor	+
2.52 g Liver	–
2.16 g Liver, 23 ppb Alachlor	+

A more sophisticated CO_2 filling scheme has been developed using a siphon tube CO_2 cylinder to fill the extraction cell. In this case, initial cooling of the cell is provided externally by an ice bath to avoid CO_2 loss before warming. This unit uses a commercial GC oven for heating a CO_2-containing reservoir upstream from the sample cell, thereby allowing larger amounts of CO_2 to be employed for the SFE. The unit has been used for the detection of spiked carbofuran in frankfurters and liver using a Enzytec (Kansas City, KS) enzyme inhibition assay (*13*).

Of course commercial SFE units can be used to continuously deliver larger quantities of SC–CO_2 for more exhaustive extraction of target analytes at high extraction pressures (*26*). We have used a Dionex Model 703 SFE unit (Dionex, Sunnyvale, CA) in conjunction with an EIA kit for the detection of the organochlorine pesticide, dieldrin, spiked in poultry fat. The Model 703 unit provides for simultaneous, parallel extraction of up to eight samples at extraction pressures up to 680 atm. Figure 5 is a schematic of the unit which consists of a pumping unit that distributes extraction fluid through a manifold for distribution to the individual cells, followed by pressure reduction through a heated restrictor element into collection vials. To integrate the sample cleanup step with SFE, the 3.5 mL cells were sealed on the exit end and filled with approximately 1.8 g of deactivated alumina (neutral, Brockman I) (*27*) for retention of the co-solubilized fat. Then 0.2 g of fat containing the pesticide was placed on top of the alumina mini-cleanup column. Extraction conditions were as follows: pressure = 250 atm, temperature = 50 °C, time = 60 min. A total CO_2 volume of 10–15 L (on an expanded basis) was adequate for eluting the organochlorine pesticide.

Collection of the extract was accomplished in an empty vial at 0 °C, followed by solubilization of the analyte with 2 mL of 0.01% Tween 20 in water. The Resi-I-Imune kit for cyclodiene pesticides (ImmunoSystems, Scarborough, ME) was used to sense the presence or absence of the dieldrin by colorimetric assay. Approximately 160 mL of the above solution was placed in each microtiter well for an absorbance reading. Figure 6 shows the results, a definitive color response for the negative control sample and a qualitative reduction in color for the extracts containing dieldrin.

Multi-vessel SFEs also can be performed using a pumpless extractor of our own design. Figure 7 is a schematic of an extractor design which can process two samples simultaneously. Liquefied CO_2 from a siphon tube source was connected to the extraction cells of the same design described previously by means of a three-way

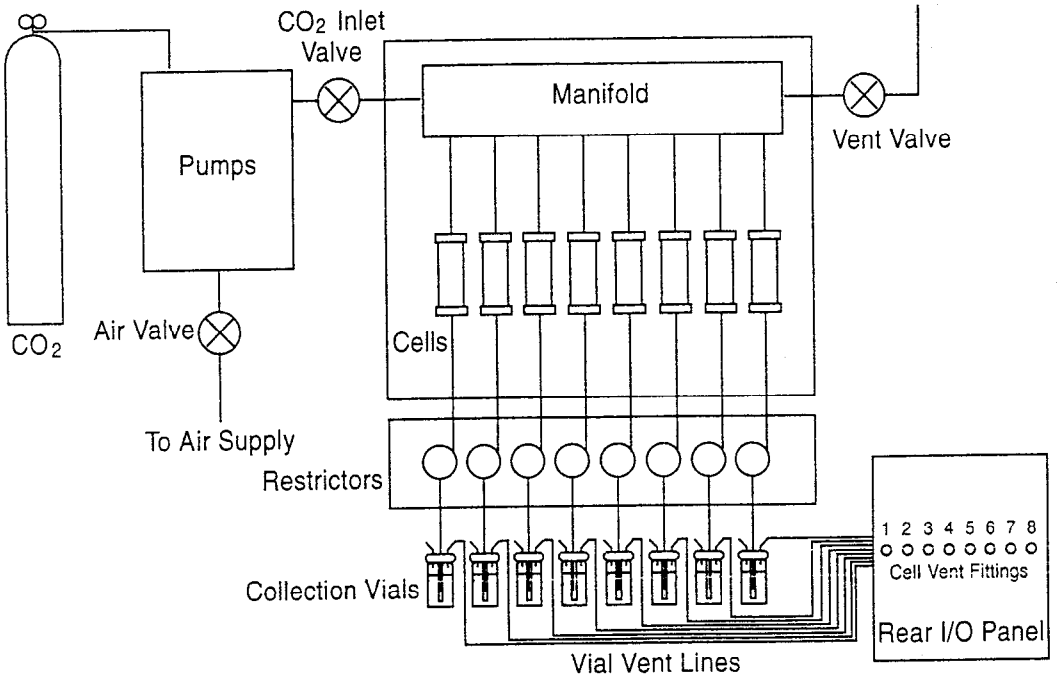

Figure 5. Schematic diagram of a continuous SFE system (Dionex 703).

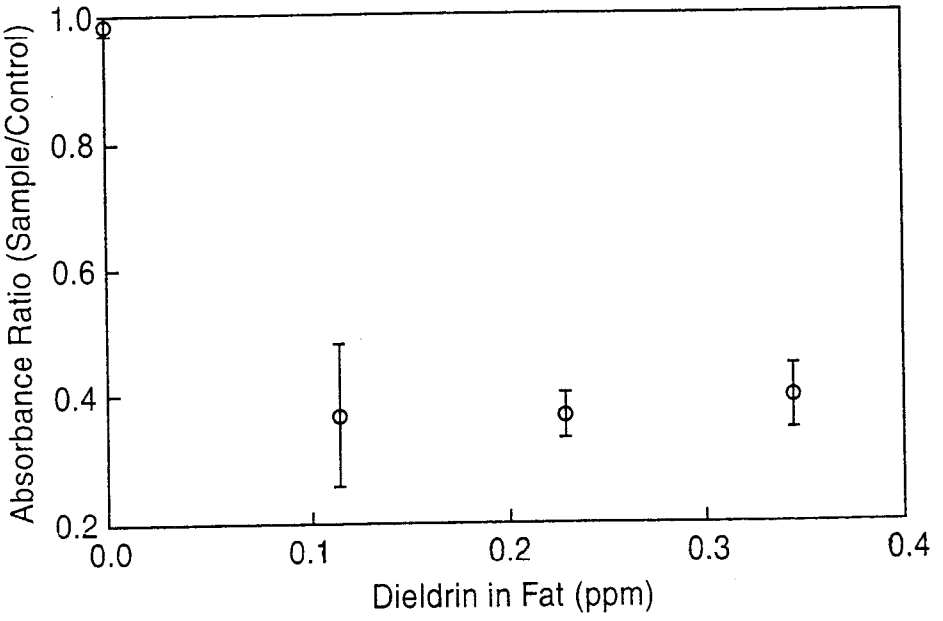

Figure 6. Detection of dieldrin in poultry fat by EIA using SFE with *in-situ* cleanup.

valve, so that either cell could be filled or jettisoned of its fluid content independently. Instead of using ice as an external coolant on the cells, the cells are immersed in a bucket of dry ice so that the CO_2 in the cell remains in its liquid state during the filling stage. After filling, the use of quick connect fittings permits the entire extraction cell assembly to be transported into a heated water bath where the extraction pressure is developed. Connection of the extraction cell device to a tandem collection assembly consisting of dual, heated micrometering valves attached to collection tubes permits extraction and collection to be affected in 40–70 min (*14*).

Figure 8 shows the results of extracting alachlor from a bovine liver sample as a function of extraction time. Figure 8 indicates that a plateau is reached after approximately 1 h or less of extraction time. Loss of analytes from the collection vessel was monitored and shown to be negligible for water or aqueous solutions containing up to 20% of methanol by volume. Use of a heated ultrasonic bath also improved analyte recovery. However, the limitations imposed by a pumpless extractor of this design, with regard to the achievable extraction pressure and/or quantity of CO_2 delivered, limits the amount of target analyte that can be extracted. A comparison of spiked alachlor extracts from bovine liver using 5 g of liver tissue mixed with 3–5 g of Hydromatrix (*28*), using methanol as a cosolvent (modifier), clearly shows the advantage of a continuous (dynamic) SFE system (Figure 9). A pumpless system using dry ice as a CO_2 source produces about a 40% yield of alachlor from the liver matrix, while the higher extraction pressure achieved using a pumpless system and liquefied CO_2 fill permits over 80% recovery of the alachlor, which is primarily due to the higher achievable extraction pressures at 50 °C (up to 600 atm). Experiments run on the Dionex extraction system with the continuous cosolvent addition module (Model 723) adding 5 mole% of methanol to the SC–CO_2 yielded quantitative recovery of alachlor from bovine liver at spiking levels up to 200 ppb at an extraction temperature and pressure of 50 °C and 450 atm, respectively. For both Figures 8 and 9, alachlor levels were determined by use of a magnetic bead-based immunoassay technique (Ohmicron, Newtown, PA).

Initial determinations of alachlor and other pesticides with the Ohmicron kits indicated a serious problem from coextracted lipid matter which interferred with quantitative EIA utilizing dynamic SFE on the Dionex unit. In contrast to the static extractions, control (blank) meat samples gave false positive readings that were above the detection limit for pesticides such as alachlor and carbofuran. Unfortunately, the previously described alumina mini-column cleanup method that can be performed *in-situ* for chlorinated pesticide assays is not applicable for the more polar pesticides. Consequently, post extraction cleanup of the extracts was explored. Three cleanup methods were tried (*14*): solid phase extraction cartridges, liquid–liquid partition, and membrane disk filtration. The main objective in the cleanup step was to remove the turbidity associated with the presence of coextracted lipids in the diluent buffer mixture in the EIA test procedure. Solid phase extraction using a C_{18} column cleanup performed on SFE extracts containing alachlor and carbofuran, yielded 103 and 97% recovery, respectively. Cleanup using an acetonitrile/hexane partitioning scheme gave 109 and 93% recovery, respectively, of the above two analytes. However, simple micro-extraction with a membrane filter, a 0.5 micron Millex-LCR membrane filter (Millipore, New Bedford, MA) proved more than sufficient yielding

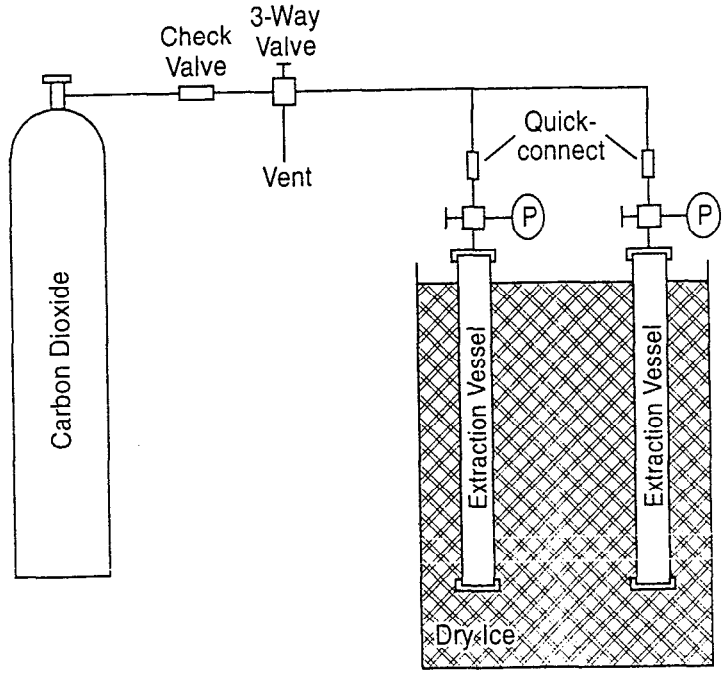

Figure 7. Schematic diagram showing a CO_2 charging system for a dual vessel, pumpless extractor.

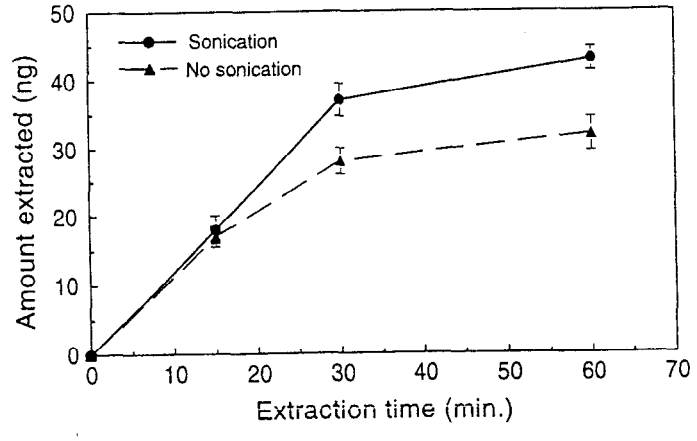

Figure 8. Extraction efficiency of alachlor from bovine liver samples using a pumpless SFE system.

100% recovery for both carbamate pesticides. This is nicely illustrated in Figure 10, where the EIA B/B_0 values between the spiked meats and blank samples were clearly distinguishable at the residue tolerance limits for alachlor (20 ppb). The general applicability of this approach is further illustrated in Figure 11 for three different pesticides assayed with the Ohmicron kits in three different sample matrices: liver, ground beef, and lard.

Quantitative results obtained with five of the Ohmicron EIA kits are presented in Table III for the pesticide analytes. These results are the mean of six individual analytical determinations for the previously mentioned sample matrices. The SFE/EIA determined recoveries and their precision indicates that all matrices are acceptable at the concentration levels of the analytes in meat or fat samples (*29,30*). Further, the minimum detection limit (MDL) achieved by the SFE/EIA method is well below the lower detection limits and residue action (tolerance) limits specified by FSIS.

Table III. SFE/EIA Results for Dynamic Extraction of Pesticide-Fortified Meat Products.

	SFE/EIA		FSIS Residue Program	
Compound	Recovery +/– SD (%)	MDL (ppb)	LDL (ppb)	Residue Limit (ppb)
Alachlor	118 +/– 13	1		20
Carbofuran	93 +/– 10	3	5	50
Atrazine	98 +/– 2	1	5	20
Benomyl	101 +/– 7	5	50	100
2,4-D	140 +/– 35	14	200	200

This indicates that the SFE/EIA methods can be used for quantitative monitoring of pesticide residues as well as the rapid screening of meat products for pesticide residue contamination.

Conclusions

The above studies indicate that SFE can be successfully integrated with a number of commercially available EIA-based kits for the detection of pesticide residues in meat and probably other associated food products. This paves the way for application of the method in food production plants where the presence of large quantities of hazardous chemicals associated with normal chemical laboratory operations cannot be tolerated. One of the major advantages of coupling SFE and EIA technologies is the speed with which toxicant contamination problems can be ascertained, thereby avoiding the expense and time associated with the routing of samples to a conventional analytical laboratory. However, even in a conventional laboratory setting SFE/EIA has the potential of reducing the number of elaborate chemical testing methods normally used in pesticide screening programs.

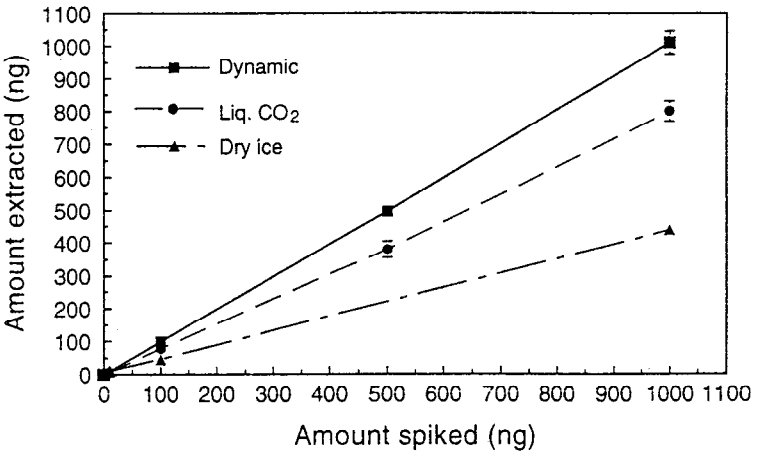

Figure 9. Extraction efficiency of alachlor from bovine liver as a function of fluid delivery system.

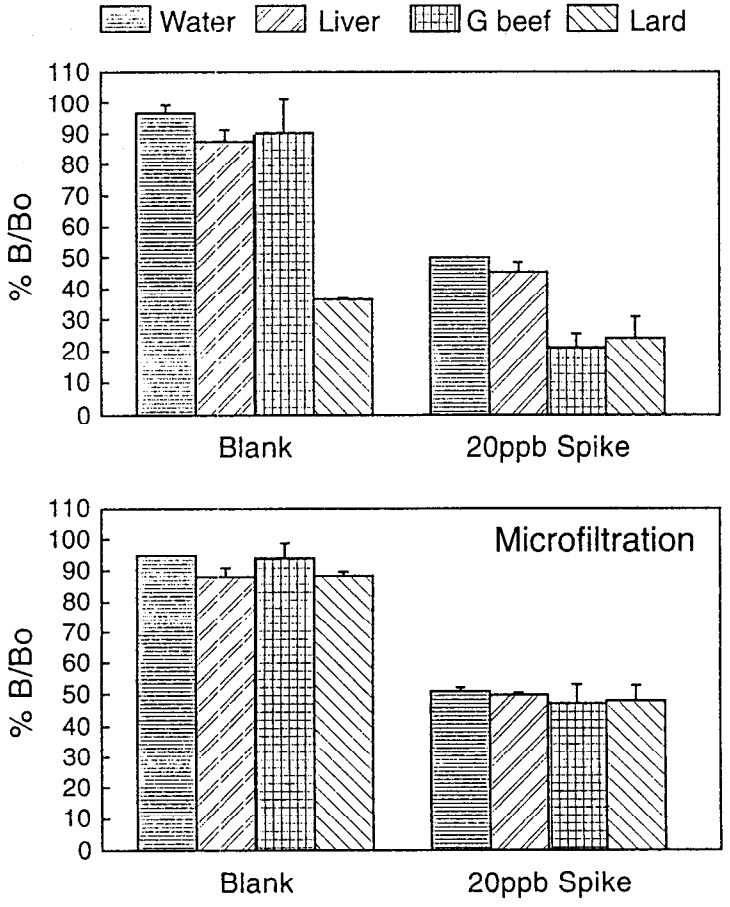

Figure 10. Effect of microfiltration of the extract from SFE on the EIA response for alachlor on different types of samples.

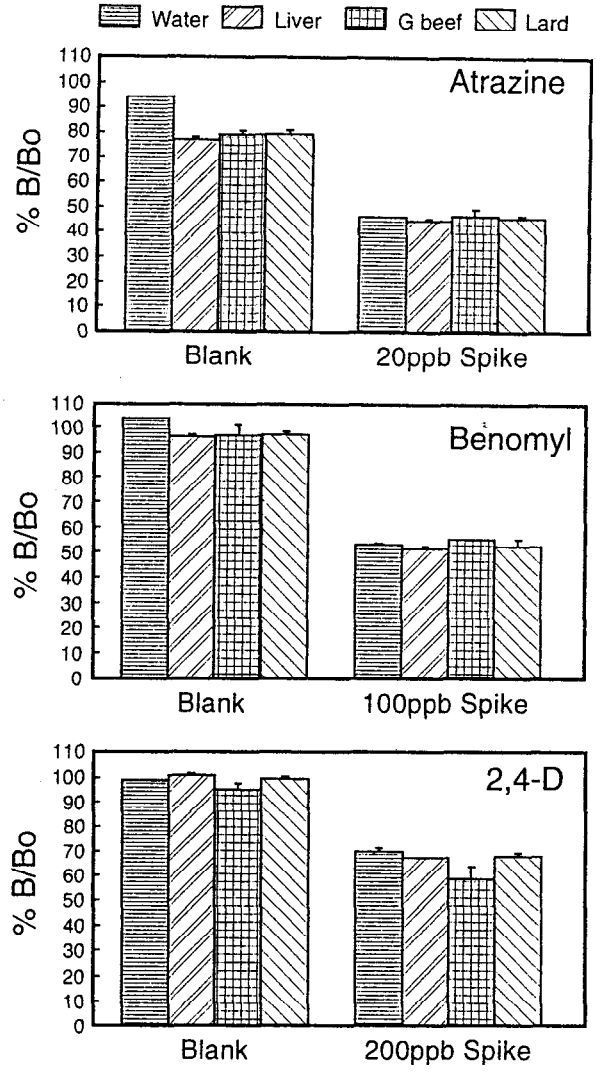

Figure 11. Results for the determination of atrazine, benomyl, and 2,4-D by EIA using dynamic SFE and microfiltration of the extract.

The above research has shown that both pumpless and conventional commercial-based dynamic SFE systems have value in residue analysis. Whereas, pumpless units appear to be limited to qualitative screening of toxicant residues in various sample matrices, the recent appearance of a commercial unit based on a thermal pump principle (*31*) opens the way for a pumpless, continuous extractor system with a dimension and size that should encourage portable use in the field.

Some sense of the impact of an SFE/EIA method on time savings and associated labor expense compared to a conventional existing chemical-based assay is provided in Figure 12. Here the key steps are listed in sequence for the determination of carbamate pesticides at trace levels in meat samples by both the proposed SFE/EIA method versus FSIS's conventional chemical protocol. The SFE/EIA assay consists of a relative simple and short extraction sequence, followed by extract filtration and centrifugation, dilution, membrane filtration, and final determination by EIA. On the other hand, the FSIS method involves sample homogenization in methylene chloride (a harmful solvent), several sequential sample treatment steps before gel permeation chromatography cleanup, which uses an eluent consisting of methylene chloride/

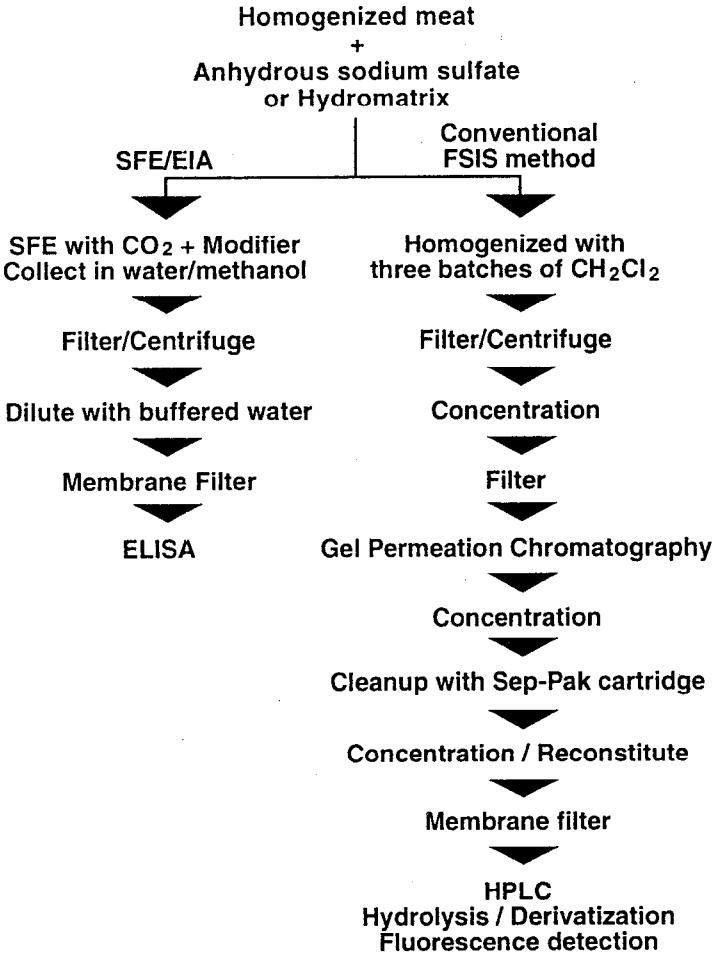

Figure 12. Comparison of SFE/EIA and conventional chemical analysis methods for the determination of carbamates in meat samples.

cyclohexane. The appropriate fraction must then be isolated, concentrated, cleaned up again on a solid phase extraction cartridge, concentrated and reconstituted, filtered through a membrane filter, before the final analytical assay is performed using a classical carbamate HPLC analysis involving analyte hydrolysis followed by derivatization for fluorescence detection (*32*).

Although the above cited example may be extreme, it illustrates that SFE/EIA is inherently a simpler method for screening food matrices and other sample types for toxicant residues, and that it has considerable potential as a quantitative technique to support and confirm the results obtained from other analytical measurements.

Acknowledgments/Disclaimer

The assistance and loan of equipment by Ohmicron and Dionex Corporations for these studies is gratefully acknowledged.

Names are necessary to report factually on available data; however the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

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RECEIVED October 3, 1995
Reprinted from ACS Symposium Series No. 621
Immunoassays for Residue Analysis: Food Safety
Ross C. Beier and Larry H. Stanker, Editors
Published 1996 by the American Chemical Society